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Review

New packing materials for protein chromatography

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Abstract

This review describes new packing materials designed for protein chromatography, covering advances in base supports and stationary phases. Base supports are classified according to their chemical composition. Since most separation media are bead shaped, typical procedures used for their preparation are also presented. In order to provide matrices combining improved chemical stability and chromatographic performances, composite materials continue to be developed, including bonded stationary phases, pore composites and mixed carriers. The different approaches to their preparation are described and characteristics that play a major role in the chromatographic process are discussed. Recently introduced materials and some of their applications under non-denaturing conditions in the different chromatographic modes are also presented.

Keywords: Reviews; Packing materials; Proteins

Contents

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1. Introduction

This review focuses on recent advances in packing materials designed for protein chromatography. Although chromatographic parameters, such as mobile phase composition, stationary phase design and column geometry, must be optimised for each protein separation, some common criteria dictate the selection of chromatographic media. First, they should be chemically and physically stable, and possess good mechanical strength to allow high flow-rates. In addition, they should not contain groups that bind proteins non-specifically, but should be easily derivatizable to allow the introduction of functional groups for interactive chromatography applications. Their ability to withstand regeneration and cleaning procedures are also important parameters. Finally, they should be produced with controllable size and pore size distributions and should be reproducible from batch to batch. Low cost, maximum throughput and high selectivity are important considerations, especially in preparative chromatography, whereas maximum efficiency is the main requirement in analytical chromatography.

During the past decades, continuing efforts have been made to improve the separation of proteins by the introduction of new packing materials. A wide variety of matrices, including organic and inorganic polymers, have been used for the design of chromatographic packings. Recently, new base materials, such as zirconia or metaphosphate, have been developed, with enhanced chemical, thermal or mechanical stability over traditional silica. New surface derivatization procedures have also been introduced in order to control non-specific adsorption and to provide new ligands with enhanced selectivity and resistance to hydrolysis. Separation media with specific features such as temperature-responsive polymers, pore-specific chemistry or artificially created sites for protein recognition were synthesised during the last years.

Interest continued in packing materials whose structural characteristics provide improved mass transfer properties and higher efficiency. Most separation media employed are bead shaped [1,2] and a wide variety of structures can be produced, as shown in Fig. 1. Porous or non-porous beaded polymer supports are mostly produced by two-phase suspension polymerization of monomers (synthetic organic polymers) but can also be obtained by suspension polycondensation and gelation (silica) or by suspension gelation and suspension cross-linking of a preformed polymer (polysaccharides). Although these traditional processes result in beads with rather broad particle sizes, appropriate variations in the polymerization conditions can afford particles with a wide variety of structural characteristics. Monodisperse particles have been produced by seeded suspension techniques, such as successive seeded polymerization [3,4] or the two-step activated swelling method [5–7]; other approaches in the use of seed particles have been developed [8–11]. Pore size distribution of a sorbent is related to the nature and the amount of porogen and cross-linking reagent in the polymerization medium. The presence of an inert solvent promotes macroreticular polymerization and the resulting porous particles are formed of agglomerated submicron nodules. More recently, polymeric porogens have been used for the preparation of macroporous monodisperse beads with a well-defined porosity [12–15].

In an attempt to decrease the interstitial porosity of columns, materials with a new geometry have been developed, allowing all solvents to flow through the separation medium rather than around it. Thus, membrane chromatography systems function in a

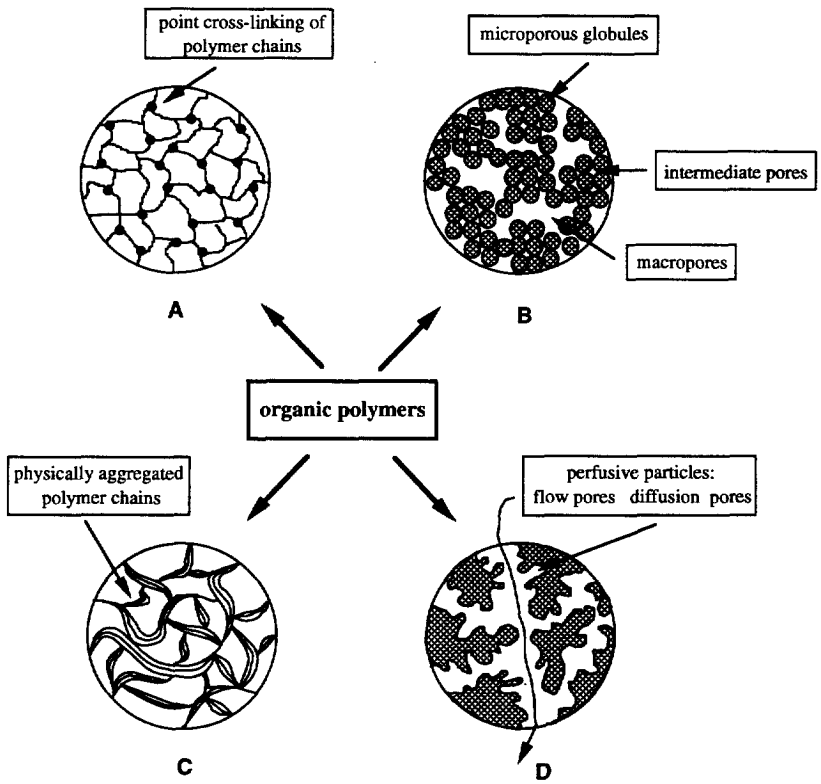


Fig. 1. Schematic representation of the physical structures of beaded porous particles for chromatographic applications: (A) homogeneous isoporous matrix; (B, C and D) heterogeneous macroporous matrices (adapted from Refs. [39,174,175]).

short and wide column, in which the packing consists of one or more membranes in series, with little or no interstitial porosity. The topic of adsorptive membranes has been reviewed recently by Roper and Lightfoot [16] and will not be detailed in this paper. In another approach to reduce the inter-particular volume of columns and to increase flow velocity, continuous macroporous media with large through channels have been recently introduced by in-situ polymerization within the chromatographic column [17–24]. In some cases, these new media compare favourably with bead-shaped particles.

In this review, current trends in packing materials for protein chromatography will be presented, covering advances in base supports and stationary phases, and evaluation of performances versus physicochemical characteristics, with selected examples that have been published mainly during the past five years.

2. General considerations

2.1. Classification of chromatographic packings and definitions

A packing material consists of a base support (core or carrier) supporting the stationary phase, which is in equilibrium with the mobile phase. The stationary phase might be the support itself or an interfacial immobilized layer.

A wide variety of materials are currently used for the chromatography of proteins. They are generally classified according to the retention mechanism that controls the chromatographic process. In size-exclusion chromatography (often referred to as gel filtration, steric exclusion or gel permeation), the separation is an entropy-controlled process, solely governed by the size of the solute, with regard to the pore size distribution of the packing. In the other

modes of chromatography, namely interactive chromatography (i.e. ion-exchange, reversed-phase, affinity and hydrophobic interaction chromatography), proteins interact with the surface of the stationary phase. The adsorption phenomenon can involve both specific or non-specific interactions. In most cases, several contributions, one of which is dominant, govern the retention of molecules. Knowledge of the packing structure and chemistry, with respect to physico-chemical and biological properties of proteins, provides useful informations regarding the choice of an appropriate separation medium.

Chromatographic packings can also be classified according to their structure. They can be divided into non-porous, superficially porous or totally porous. The terms rigid, semi-rigid or soft packings are related to their mechanical strength, which depends strongly on their ability to shrink or swell in the presence of certain solvents. Soft gels (xerogel) exhibit poor mechanical stability and can withstand pressures up to 3–5 bars only. Semi-rigid materials can be operated under medium pressure (15–30 bars), whereas rigid materials (aerogels) can be operated under high pressure (200–300 bars). Aerogels are not affected by solvents and exhibit a permanent porosity.

Furthermore, as a wide variety of materials can be used for the design of chromatographic packings, one may also divide them according to their chemical bulk composition, i.e. organic or inorganic. Materials that combine the properties of two or more components are referred to as composite materials.

2.2. Chromatographic behaviour of proteins

The theories and mechanisms of protein adsorption at interfaces have been extensively reviewed [25–27]. The behaviour of proteins in a chromatographic system is determined by many factors, including their stability, conformational flexibility, charge surface and hydrophobicity. Obviously, it depends also on the nature of both the stationary phase and the mobile phase as well as on the operating parameters. Recent approaches in qualitative and quantitative analysis of proteins and peptides by HPLC, focusing on conformational and dynamic aspects of the protein adsorption/desorption mechanism, have been reviewed by Schöneich et al. [28].

Numerous mathematical models have been developed to predict the behaviour of a given protein at a given solid–liquid interface. So far, the more rigorous models are those that take into account both surface interactions and mass-transfer considerations [29–31]. However, more comprehensive models should be used to describe the adsorption of two or more proteins on a surface, since this more complex event may involve competitive adsorption. The best known example of competitive adsorption is the "Wroman effect", mainly observed with blood and plasma proteins. The "Wroman effect" provides evidence that more abundant proteins of low binding affinity, first adsorbed after a short period of time, are progressively displaced by less abundant proteins of higher binding affinity. Generally speaking, parameters to be considered for predicting differential protein adsorption are relative bulk solution concentrations, diffusion coefficients and affinity for the surface, which may evolve with time through conformational changes.

Adjustments of parameters have to be optimized for each separation, with regard to the binding characteristics of protein and to its intended use, i.e. the final purity required. Over the past years, several interesting reviews appeared, most of them describing advances in sorbents for preparative chromatography through selected applications [32–37].

Some general principles and concepts govern current research and the use of chromatographic media. Optimum resolution (i.e. selectivity and efficiency) can be achieved by decreasing non-specific binding with the surface, increasing the specificity and the accessibility of adsorption sites and reducing the effect of diffusion within the chromatographic bed. Schematically, adsorption and desorption of proteins are determined by (i) convective transport of solute molecules between particles, (ii) diffusion of the molecules from the bulk solution to the external surface of particles, (iii) diffusion within the porous structure and (iv) attachment to the active sites on the surface. Attachment should be reversible, by modifying the elution conditions. As the adsorption site density and/or free adsorption energy increase, the probability of desorption decreases. Since most proteins can undergo various structural

and conformational transformations, leading to changes in their biological activity, mild elution conditions are required and extremes of pH or temperature should be avoided. Furthermore, operational times must be minimized, since the probability of degradation and contamination (viruses, pyrogens, bacteria) increases with time.

Extraparticle convective transport may be improved by using small non-porous and regularly shaped particles or continuous beds. In porous materials, pore diffusion is often rate-limiting. Thus, the matrix should possess a narrow pore size distribution, with pores large enough to facilitate penetration by proteins. In interactive chromatography, better resolutions are obtained when pore accessibility is not restricted ($R_H/R_p<0.1$, with R_H= hydrodynamic radius of protein and R_p=pore radius).

Nevertheless, it should be kept in mind that the higher the pore size, the lower the specific surface area and, consequently, the lower the binding capacity. An alternative to enhance pore mass transfer without reducing the capacity too much has been accomplished with gigaporous packing [38], including perfusive materials [39,40] in which convection dominates on diffusional transport, continuous rods [17–24] and pore matrix composites [41–44]. In pore matrix composites, the presence in the pores of flexible polymer chains, with relatively free movements and, thus, less hindering solute diffusion, contributes to a decrease in mass transfer resistance within the stationary phase while keeping capacity at a high level.

3. Advances in base packing materials

In most cases, a description of column packings includes two aspects; the base support and the stationary phase that is chemically or physically immobilized onto the core and carries the chromatographic functions. In some packings, the composition of the carrier and of the stationary phase are identical.

Numerous packing materials, mostly those based on hydrophilic polymers, are produced by linking the chromatographic functionalities directly or via a short spacer onto the core. In contrast, composite supports are obtained by coating a non-inert core particle with a polymeric layer, prior to the introduction of functional groups.

The base support plays a determinant role in the mechanical, chemical and thermal stability of packing materials. Chromatographic performances are strongly related to its structural characteristics as well as to the physico-chemical properties of the stationary phase, since protein adsorption/desorption mechanisms occur at the surface. Ideally, a stationary phase should be chemically resistant and should effectively shield the core from solvent degradation or non-specific interactions with biomolecules. According to their chemical composition, base materials may be divided into inorganic polymers, organic polymers and composite materials.

3.1. Inorganic polymers

Undoubtedly, silica is the most widely used chromatographic material, being available in a wide range of particle sizes and porosities (pore diameters of 5–400 nm) [45]. The most common porous silica particles are obtained by polycondensation of silicic acid, followed by thermal treatments. Gigaporous silica, with pore diameters >1000 nm, have been produced by widening the pore size of mesoporous silica following a calcination procedure in the presence of organic salts [46]. Recently, a continuous silica column, with a mean pore diameter of the order of 2000 nm, has been synthesised [47]; however, to our knowledge, this material has not yet been used for the purification of biomolecules.

Although silica exists in various forms, amorphous forms are generally used for chromatographic applications. Silica is very stable under pressure and can easily be derivatized, to introduce functional ligands. Pure silica particles are amorphous and possess a bulk composition of (SiO_2, H_2O_n). The silicon–oxygen bonds at the surface can be siloxane bonds (Si-O-Si), isolated silanol groups (Si-OH) or associated silanol groups through hydrogen bonds (Si-O· · ·HO-Si) when close together. Nevertheless, most commercial silicas are not similar and often possess an heterogeneous surface. They may contain impurities and exhibit variations in the type and reactivity of their silanol groups [48].

Some drawbacks preclude the use of unmodified

silica for the chromatography of proteins. Silica is unstable at mild alkaline pH values and dissolves drastically above pH 8. Furthermore, non-specific interactions occur above pH 4 between deprotonated silanol groups (pK_a=6–8) and the basic part of biomolecules. These drawbacks can be limited by decreasing the activity of the silanol groups. This can be achieved by using silica with enhanced purity and with a high population of internally bonded or associated silanol groups, which are less acidic than free silanol groups [49,50]. An effective minimization in surface activity can also be achieved by chemical modification of silanol groups with monomeric or polymeric silanes. However, at low pH, the siloxane bonds, if not sterically protected by bulky groups, tend to hydrolyse, causing changes in chromatographic performance.

Due to the chemical instability of silica, the synthesis of novel chromatographic media that combine high mechanical strength with good chemical stability continues to generate interest. This explains the increasing attention directed towards other metal oxides, such as alumina (Al_2O_3), titania (TiO_2) or zirconia (ZrO_2).

Metal oxides exist in many amorphous and crystallographic forms. Their chemical stability is higher than that of silica. Alumina is chemically more resistant than silica and dissolves only above pH>12 and under pH<3. However, its porous structure is generally heterogeneous and alumina columns exhibit lower efficiency than silica columns. Zirconia and titania possess excellent mechanical properties and chemical stability, allowing the preparation of particles able to withstand high pressures (up to 500–600 bars) and extreme pH conditions (pH 1–14). The complex properties of zirconia and its use in chromatography have been well documented by Nawrocki et al. [51]. Since particle size and pore size characteristics are important for good chromatographic separations, various methods to prepare spherical titania or zirconia microbeads have been investigated, either by a sol-gel process [52] or by polymerization-induced colloidal aggregation [53,54]. Heat treatment results in the development of the porous structure and smaller particles ($\simeq$6 μm) are obtained by the polymerization-induced colloidal aggregation method.

Alumina, titania and zirconia surfaces are less acidic than silica surfaces. According to Kurganov et al. [55], isoelectric points of oxides decrease from ZrO_2 (pI=10–13), Al_2O_3 (pI=7), TiO_2 (pI=5) to SiO_2 (pI=3). This property allows the separation of some basic species that would otherwise be highly retained on silica [52,55]. Furthermore, in contrast to silica, these oxides show high ligand-exchange behaviour, due to the presence of strong Lewis acid sites at their surfaces. They can interact with Lewis bases, such as fluoride, phosphate ions or carboxylic acids, and also with the carboxyl groups of proteins. Protein separation can be achieved by using a mobile phase containing a strong Lewis base at well defined concentrations [51,56]. However, passivation procedures can also be applied to suppress reactive sites, as mentioned for silica.

Crystalline pyrophosphates ($Me_2P_2O_7$) and metaphosphates ($Me_x(PO_3)_n$) (with Me=Ca, Mg, Sr, Mn...) have recently been examined for the separation of proteins and compared with calcium phosphate hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [57,58]. Hydroxyapatite has well known unique separation properties; at neutral pH, basic proteins (pI>7) are mainly adsorbed via electrostatic interactions with negatively charged phosphate ions, while carboxylic groups of acidic proteins complex specifically with calcium sites [59]. While crystalline hydroxyapatite is rather fragile and stable over a limited pH range (5–10), crystalline metaphosphates display excellent mechanical, thermal and chemical stability. The charge distribution on the surface of pyrophosphate, metaphosphate or hydroxyapatite depends more on pH than on the structure of the crystal. Thus, at neutral pH, all of these materials exhibit similar behaviour towards basic proteins. In contrast, acidic proteins are retained according to different mechanisms, depending on the nature of the metal ion (Me) and on the geometric arrangement in the crystal. Such materials may provide useful chromatographic sorbents for the specific purification of proteins, without further modifications.

3.2. Organic polymers

3.2.1. Polysaccharides

The first organic polymers to attract interest were natural polysaccharides, including agarose, cellulose, cross-linked dextran and, to a lesser extent, cross-

linked amylose and starch. These materials are produced with a suitable porosity, are stable over a wide pH range (pH 3–13) and are able to withstand alkaline washing. In addition, they possess a high content of hydroxyl groups available for activation and derivatization, they are hydrophilic and generally do not interact with proteins. However, under extreme conditions, they may exhibit weak ionic or hydrophobic properties. Specific interactions with proteins have also been reported, e.g., haemoglobin is able to recognise α-1,4 glycosidic links in amylose [60], and the OH groups of polysaccharides are targets for the C3 protein of blood plasma [61].

The main drawback of polysaccharides is their poor mechanical strength, related to their swelling ability. The extent of polymer swelling depends on the chemical nature and on the concentration of polymer in particles and it decreases as the degree of cross-linking increases. Gel rigidity is also closely associated to the chain length of the cross-linking agent.

Cellulose is the most abundant hydrophilic polysaccharide. It has long been available in fibrous or microgranular forms. However, these irregularly shaped particles are not suitable for current chromatographic processes. Procedures are now available for the preparation of spherical beads. Bead-shaped cellulose or agarose particles are classically obtained by suspension-gelation. Both agarose and cellulose exhibit crystalline regions where chains are aggregated and form a physically stabilised porous network. These materials are semi-rigid (hybrid xerogel–aerogel) and modern technologies allow the preparation of macroporous particles capable of sustaining relatively high flow-rates without any back pressure problems. For instance, macroporous cellulose beads (Divicell, 80–200 μm diameter), prepared without chemical cross-linking, have been reported to withstand high flow-rates, i.e. up to 2000 cm/h [62]. Recently, non-cross-linked superporous agarose beads, characterized by normal diffusive pores and wide through pores, have been prepared by a double emulsification procedure. Although rather large (300–500 μm), these new particles exhibit performances close to those of much smaller diffusive particles, due to the presence of large flow pores. In addition, the plate height was found to be independent of the flow-rate, up to 130 cm/h [63].

The mechanical stability of cellulose or agarose particles can be improved by chemical cross-linking, using epichlorohydrin, bisepoxides or divinylsulfone. Cross-linking does not significantly affect the macroporosity but may lead to undesirable interactions with proteins. As an example, the thiophilic nature of divinylsulfone-cross-linked agarose has been recently evidenced in the presence of serum immunoglobulins [64].

Individual dextran chains are flexible and soluble in water. The procedure used to prepare dextran particles is similar to that used for agarose. However, as dextran solutions do not exhibit any sol-gel transition behaviour, a cross-linking reagent must by added to the water/oil (w/o) suspension. The resulting particles are stabilized by point cross-linking of dextran chains; however, increasing the stability of the polymer network by increasing cross-linking significantly reduces the porosity. Cross-linked dextrans are typical examples of soft gels (xerogel) whose rigidity and porosity are strongly related to their swelling ability.

Other organic materials are based on synthetic polymers. Their main advantage over silica is an increased pH stability. Most of them are also more resistant to pressure than polysaccharides. Disadvantages in comparison to inorganic materials are lower pressure tolerance, swelling changes that may occur in the presence of organic solvents, broader pore size distributions and decreased efficiency.

3.2.2. Polyacrylamides

Soft gels based on dilute polyacrylamides were first obtained by copolymerization of acrylamide and methylene bisacrylamide in a w/o suspension. As described for dextran-based materials, the degree of cross-linking controls both the permeability and the rigidity of the matrix. Polyacrylamides are stable in acidic media but rapidly hydrolyse to polyacrylic acid above pH 10. Research work has been carried out to improve their properties, through changes in the chemical composition and concentration of monomers and cross-linkers [65]. Significant improvements in the flow properties have been obtained with continuous beds, which result from the polymerization directly into the chromatographic tube of monomers such as methacrylamide and piperazine diacrylamide [20,24,66]; flow velocities as high as

2000 cm/h can be used while keeping the back-pressure at a low level.

In the polyacrylamide group, one may also include poly(vinyldimethylazalactone–co-methylene bisacrylamide) beads (Emphase), since azalactone groups can be derivatized by reaction with nucleophilic molecules, leading to polyacrylamide derivatives. As examples, a hydrophilic size-exclusion medium was prepared by reacting glucosamine [67], while IgG affinity supports were obtained after reaction with protein A or G [68]. This porous material (pore size 100–1000 Å) is stable both in aqueous and non-aqueous solutions and allows linear flow-rates of up to 3000 cm/h.

3.2.3. Polyacrylates

Spherical poly(hydroxyethylmethacrylate) (HEMA) sorbents, introduced twenty years ago, are prepared by suspension copolymerization of 2-hydroxyethylmethacrylate and ethylene dimethacrylate [69]. When a large amount of cross-linking monomer is used, the rigidity of particles is high and permits their use at high pressure (up to 200 bars). Such HEMA particles exhibit a strong resistance to hydrolysis (stability pH range: 2–12), a low degree of swelling in water as well as in organic solvents, and a high surface density of hydroxyl groups. Without further modifications, HEMA beads can be used either in size-exclusion or in hydrophobic interaction chromatography of proteins, due to their mild hydrophobic character. Reduction of hydrophobicity can be achieved by different methods, including treatment with epichlorohydrin and subsequent hydrolysis [70].

Monodisperse macroporous oligo(ethylene glycol)dimethacrylate particles have been recently prepared by seed polymerization [10]. Ogino et al. [10] showed that hydrophilicity increases as the number of oxyethylene units ($n=2$, 3, 4) increases. When $n=4$, the hydrophilicity was similar to that of HEMA sorbents. However, the main disadvantage of this polymeric media over HEMA material is probably the lack of derivatizable hydroxyl groups on the surface.

Novel separation media, based on continuous rods or uniformly sized porous glycidylmethacrylate–ethylenedimethacrylate polymers, have also been described. The epoxide groups can be simply hydrolysed to afford diol functionalities; the surface thus obtained is sufficiently hydrophilic to avoid non-specific adsorption of proteins. Epoxy groups have also been derivatized using classical reactions or pore-size-specific functionalization to provide ion-exchange materials [18,22] or restricted access media [71,72].

3.2.4. Polyvinyl polymers

Most polyvinyl polymers are based on polystyrene–divinylbenzene copolymers (PS–DVB). Through recent progress in polymerization processes, it is possible to prepare rigid beads with a high degree of cross-linking, capable of withstanding pressures of up to 300 bars. Obviously, the pressure limit depends to a large extent on the porous structure. Monodisperse macroporous PS–DVB particles are mainly produced by seed polymerization and related processes. Recent additions to PS–DVB media are continuous macroporous rods [21,23,73] and perfusion packings [39,40]. In both cases, the mobile phase flows through the material rather than around it and the mass transport of molecules is enhanced. Compared to silica, rigid polystyrene matrices exhibit the advantage of being ion-free and stable over the whole pH range. However, the hydrophobic character of PS–DVB matrices is a serious limitation to their direct use in biochromatography, except in the reversed-phase mode, and surface modifications must be used to increase their polarity.

Poly(vinyl phenol)-based particles have been recently introduced, by polymerization of 4-*tert.*-butoxycarbonyloxystyrene followed by removal of 4-*tert.*-butoxycarbonyl groups. Compared to PS–DVB beads, they are more polar, although still hydrophobic, and exhibit negligible swelling changes in organic solvents such as tetrahydrofuran (THF) [9].

Other carrier media based on poly(vinyl alcohol) (PVA) are obtained by suspension polymerization of poly(vinyl acetate). The acetate groups are subsequently hydrolysed to provide a more hydrophilic material. However, hydrophobic retention has been evidenced with some proteins, such as chymotrypsinogen or bovine serum albumin, in the presence of salts ($NaCl \geq 1$ *M*). Furthermore, PVA particles suffer from poor mechanical strength. A more resis-

tant material has been prepared by repeated cycles of freezing and thawing of original PVA particles [74].

4. Composite materials

Base materials are often responsible for non-specific adsorption of proteins. In addition, some of them are chemically unstable or display poor chromatographic performance under certain operating conditions. This explains why intensive research has been directed towards composite materials. The primary approach in composite design consists of associating the rigidity of a carrier (e.g. silica or polystyrene) together with the biocompatibility and the chemical stability of another component, acting as the stationary phase. Another important goal is to improve chromatographic performance, with regard to the structural characteristics of materials. Composite packing may be divided into three main categories, including (i) surface bonded stationary phases, (ii) pore–matrix composites and (iii) mixed carriers. In spite of this classification, combined forms can exist. For example, surface bonded polymers can be regarded as surface bonded composites (in bad solvent) or as pore–matrix composites (in good solvent), depending on the ability of adsorbed polymeric chains to shrink or to expand into the pores. Schematic representations of composite materials are given in Fig. 2.

4.1. Surface bonded stationary phases

Ideally, bonded phases should be uniform and sufficiently dense to prevent interactions between the protein and the carrier surface. Depending on the starting materials and on the intended applications, the coating process should be optimized in order to obtain a bonded layer that is either thin enough to retain the porous structure of the starting materials or presenting flexible arms protruding into the solution. Irrespective of chemical composition considerations, the structure and conformation of stationary phases play important roles on chromatographic properties and some of their characteristics are directly related to the immobilization process, i.e., the number of contact points with the base particle, polymer loading, uniformity of the adsorbed layer, degree of cross-linking and pore filling. Surface bonded stationary phases can be prepared by various ways.

4.1.1. Chemical modification

This can be achieved by linking functional groups directly to the surface, leading to monolayer bonded phases. The most efficient shielding of surfaces is obtained with polymer layers, resulting from the covalent grafting of a preformed polymer or from the graft polymerization of suitable monomers. Such polymer-modified materials are often referred to as "core shell graft". The "pellicular" category includes sorbents in which the core shell is cross-linked and/or forms stable multiple bonds with the core surface. Although originally restricted to non-porous materials, the term "pellicular" has been extended to porous particles that have a thin polymer layer applied to the surface, whereas micropellicular sorbents still refers to small non-porous particles (diameter of the particle < 10 μm). The term "tentacle" was first used to describe ion-exchange materials, obtained by one-end grafting of linear polymer chains onto the solid core. Some of these tentacle supports have been proved to exhibit higher mass-transfer, selectivity and binding capacity compared to more conventional ion-exchangers, due to improved site accessibility and, thus, lower protein distortion [75].

Most grafting reactions on silica or on other metal (Me) oxides involve the use of organosilanes that react with hydroxyl groups present on the surface. According to Schindler and Schmidbaur [76], the order of stability of Me-O-Si bonds is: Si-O-Si>Zr-O-Si>Ti-O-Si>Al-O-Si and numerous attempts to improve hydrolytic stability, using hindered or polymeric silane, have been reported [45,77–80]. During the last five years, most silica packings designed for size-exclusion or interactive chromatography have been produced by grafting polymers containing silane functionalities onto native surfaces or by grafting polymers onto previously derivatized surfaces. Various methods for silica surface activation have been examined recently by Mingalyov and Fadeev [81]. Recently introduced silica-based packings resulted from covalent grafting of poly(styrene–co-vinylsilane) [82], poly(ethylene oxide)monomethacrylate [83], dextran and aminoethyldextran [84], poly(N-diethylaminoethylacryl-

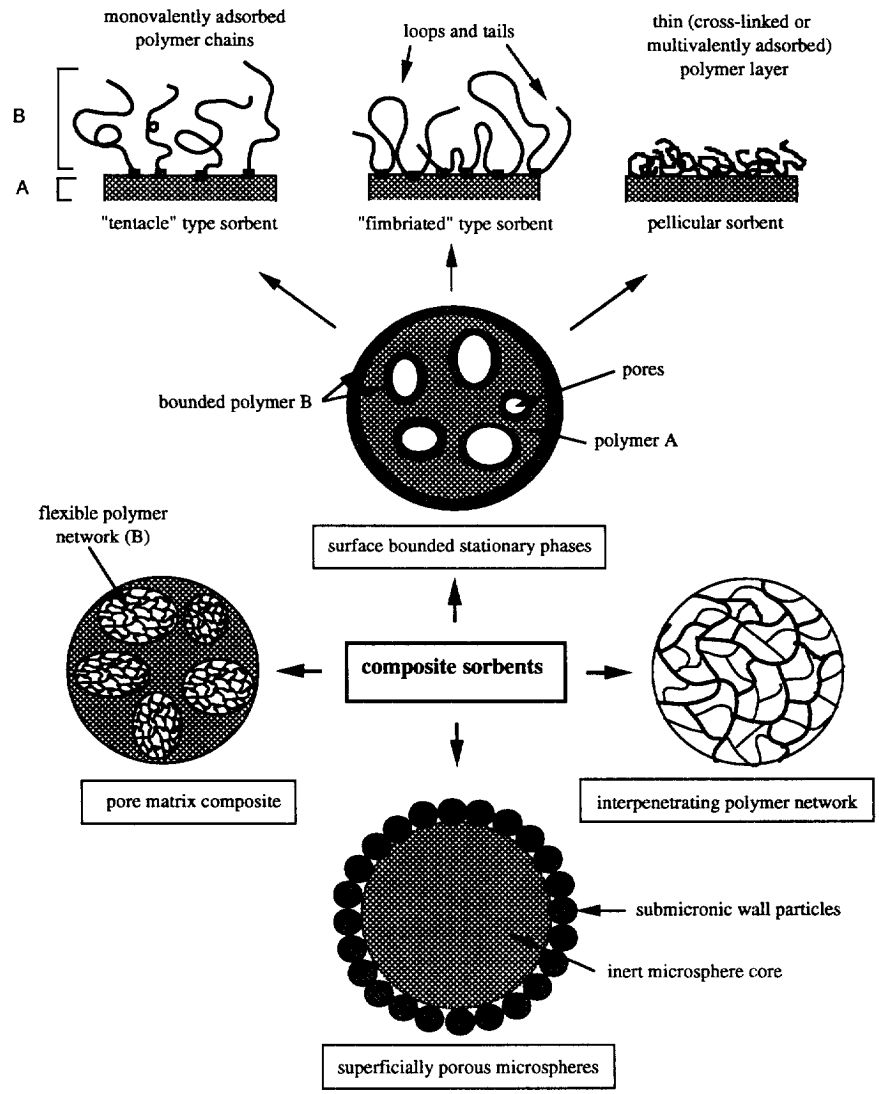


Fig. 2. Schematic representation of composite sorbents (adapted from Refs. [1,104,118]).

amide) [85,86], polyvinylpyrrolidone [87] or trifluorostyrene–vinylmethyldiethoxysilane copolymer [88]. Improvement of the hydrolytic stability and prevention of electrostatic interactions with proteins can be achieved by a combination of steric shielding with polymers, multivalent attachment of the bonded layer and cross-linking. For instance, Kurganov et al. [89] investigated the modification of porous silica or other oxides by covalent binding of poly(styrene–co-vinylsilane) and found that additional cross-linking of the bonded polymer phase significantly improves the pH stability of packings. In addition, ionic interactions of proteins with residual silanols can be minimized by controlled introduction of positive charges onto the grafted polymer; Petro et al. [84] showed that silanol groups on a silica surface are more effectively masked by grafting aminoethyl dextran rather than unmodified dextran, since, in the first case, steric shielding is combined with electrostatic compensation of SiO^-.

Another way to increase the hydrolytic stability of silica packings consists of grafting a silicone mono-

layer onto the silica surface, using 1,3,5,7-tetramethylcyclotetrasiloxane as a silicone monomer, before attaching functional groups. These phases have excellent pH stability (pH 2 to 11) [90] and have been used recently to prepare a mixed hydrophobic–hydrophilic material for the direct analysis of biological samples [91,92].

Only a few organic polymer-based packings modified with covalently bound polymer layers have been introduced recently [6,93]. Because polystyrene does not contain reactive functions, preliminary activation steps are necessary, most of them being based on Friedel–Craft reactions. Although recent methods to increase the reactivity of PS–DVB beads have been investigated, such as copolymerization of divinylbenzene with more reactive styrenic monomers [9,23,94], the simplest way to modify polystyrene remains the physical adsorption of polymers.

Recently, several polymeric stationary phases with particular chromatographic features have been described. Hosoya et al. [95] investigated hydrophobic macroporous polystyrene beads with a hydrophilic external layer of poly(glycerolmethacrylate), for the separation of mixtures containing proteins and hydrophobic drugs. The hydrophilic external layer was introduced during the final polymerization step of PS–DVB particles and was shown to prevent protein adsorption, whereas the selectivity of polystyrene towards small hydrophobic solutes was preserved.

Poly(N-isopropylacrylamide) (PNIPAM), a temperature-responsive polymer, was coupled with previously aminated porous glass beads. This support was not tested for biopolymer chromatography but interesting results were obtained in the size-exclusion chromatography of dextrans; the elution time of dextrans increased on increasing the temperature from 25 to 32°C. This was attributed to an increase in the pore size of the material, due to the coil–globule transition of the end grafted PNIPAM above its phase transition temperature [96]. PNIPAM was used later on to modify porous poly(ethylene-dimethacrylate) beads, either on the internal or the external surface of particles [97]. The externally modified packing showed an increase in bovine serum albumin (BSA) retention above the phase transition temperature of PNIPAM, due to hydrophobic interactions with the "deprotected" surface of core particles.

A molecular imprinted stationary phase for metal chelate chromatography of RNase A was prepared by (i) derivatization of silica with methacrylate groups, (ii) polymerization of N-(4-vinyl)-benzyl iminodiacetic acid in the presence of copper ions and coordinated RNase A and (iii) removing the protein. In the presence of metal ions, the resulting support showed an unusual affinity for RNase A [98].

4.1.2. Physical adsorption of polymers

Physically adsorbed polymer layers can be prepared by polymerization of a monomer that was previously adsorbed on the surface or by depositing a preformed polymer. This method is simpler than the former one due to the fact that no surface derivatization procedures are needed and, thus, no restrictions are linked to the surface chemistry. However, if the adsorbed polymer is soluble in chromatographic mobile phases, further cross-linking is required, either between polymer chains or between polymer and surface, in order to increase the stability of the coated layer. Most of these materials belong to the "pellicular" category, as previously defined. Another important point to consider is the size of the polymer with regard to the pore size of the base particle and the affinity of polymer for the surface in the adsorption solvent. Higher affinity will promote better adhesion of polymer onto the surface and, in most cases, a more homogeneous coating is expected. On the other hand, different polymer distributions across modified surfaces are obtained, depending on the method of polymer immobilization.

A first coating method consists of depositing a polymer or a monomer on the particle surface, followed by solvent evaporation and cross-linking. Recent examples include the deposition of polybutadiene (PBD) onto silica, zirconia and alumina [89,99–101] or silica modifications with polymethacrylate-based polymers or copolymers [83,102,103]. According to Hanson et al. [99], a PBD-coating prepared by polymerization of oligobutadiene onto porous silica does not result in a homogeneous film but rather in an inhomogeneous pore filling. Proteins are eluted from PBD–Si columns according to a reversed-phase mode, but they are irreversibly adsorbed on PBD–zirconia; all of the Lewis acid sites on the surface of zirconia are not completely blocked and cause strong interactions

with carboxylate groups of proteins. This drawback has been overcome by using a mobile phase containing an organic solvent, phosphoric acid and displacing salts [100,101]. In a cation exchanger prepared by covering silica with sulfonated poly(N,N′-dimethylacrylamide–co-glycidylacrylate), residual silanols groups were also found to participate to the retention mechanism of basic proteins [103].

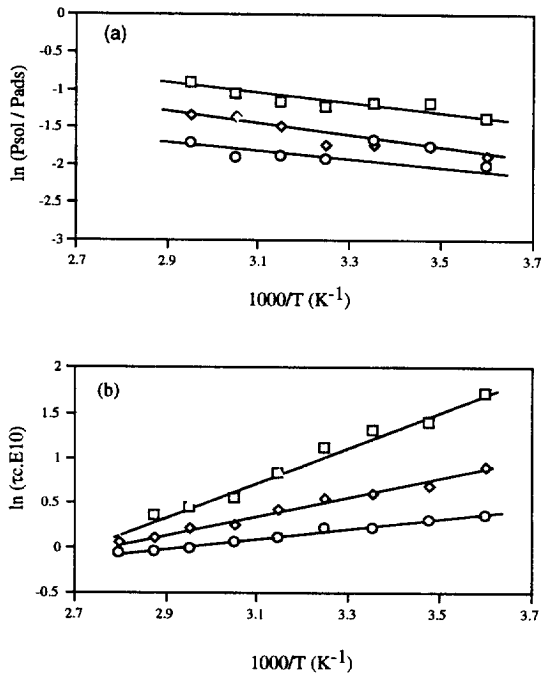
A second way to prepare bonded phases consists of a three-step procedure: (i) adsorption of polymer and/or monomer from solution, (ii) removal of the non-adsorbed polymer by filtration and (iii) cross-linking the adsorbed layer. This technique involves the use of polymers capable of interacting strongly with the core surface via hydrogen bonds, ionic or hydrophobic interactions. Such a method has been extensively used for surface hydrophilization and provides useful materials for size-exclusion or affinity chromatography after derivatization. Among recent examples, one can mention the adsorption of polyglycerol [104], poly(vinyl alcohol) [105,106] or hydrophobically modified dextran [107] onto PS–DVB particles, adsorption of DEAE–dextran [108] or poly(N-vinylpyrrolidone–co-vinylchloroformate) [109] onto silica or of carboxymethyl–dextran [110] onto zirconia. The conformation of the polymer layer depends on the number of contact points between the polymer and the base material and on polymer solvation. According to the definition proposed by Varady et al. [104], some of these materials should be regarded as "fimbriated" matrices, if long hydrophilic segments, located between two adsorbing units, extend in solution as a loop or a queue and take the high flexibility of polar filaments [104]. For instance, we showed by electron paramagnetic resonance (EPR) spectroscopy measurements that hydrophobically modified dextrans adsorbed onto PS–DVB particles form loops extending in solution; the higher the hydrophobic group content of modified dextran, the higher the number of loops but the smaller their size and their mobility (Fig. 3). In addition, we showed that the non-specific adsorption of BSA is strongly dependent on the conformation of the adsorbed layer (Fig. 4) [111]. Thus, as the content of hydrophobic groups in the adsorbed dextran increases (at similar dextran coverage), a more densely packed layer, less permeable to proteins, is obtained.

4.2. Pore matrix composites

Pore matrix composites combine the high selectivity of a soft gel with the pressure stability of a rigid matrix. In these composites, the pores of a macroporous rigid material (pore size 2000–6000 Å) are filled with a soft and porous polymer network. After functionalization, good accessibility of the bound ligand can be achieved, partly due to the flexibility of the polymer network. These materials generally exhibit high binding capacity even at high linear velocity. Various applications of pore matrix composites have been described recently: Hyper D supports, designed for ion-exchange chromatography, have been prepared by incorporating an ion-exchange hydrogel in the pores of a rigid polystyrene–silica skeleton [44]. Other materials have been obtained by filling the macropores of cross-linked agarose [41], polyhydroxymethacrylate [42,43], silica and carbon [112] with dextran. The dextran network was stabilized in the pores either by chemical binding onto the pore surface or by chemical cross-linking. The amount of dextran, as well as the molar ratio of cross-linking reagent to dextran, must be adjusted to match the porosity and selectivity. However, proteins that are small enough to penetrate the dextran network can interact non-specifically with the internal pore surface. In order to overcome this drawback, an alternative method consists of modifying the pore surface prior to filling the pores. Petro et al. [112] used a derivatized silica to prepare a pore matrix composite but showed that the previously bonded monolayer did not prevent the adsorption of small analytes. It is probable that the deposition of a first, densely cross-linked, hydrophilic polymer layer prior to pore filling would be more efficient for avoiding non-specific adsorption.

4.3. Composite carriers

A first class of composite carriers includes homogeneous polymer networks. They are formed by two inter-penetrating polymeric components, homogeneously distributed within the particles. Composites of this type are not new and the first one was Ultrogel AcA, composed of polyacrylamide and agarose. The agarose matrix gives the particles their mechanical strength, while polyacrylamide provides high resolution in size-exclusion chromatography.



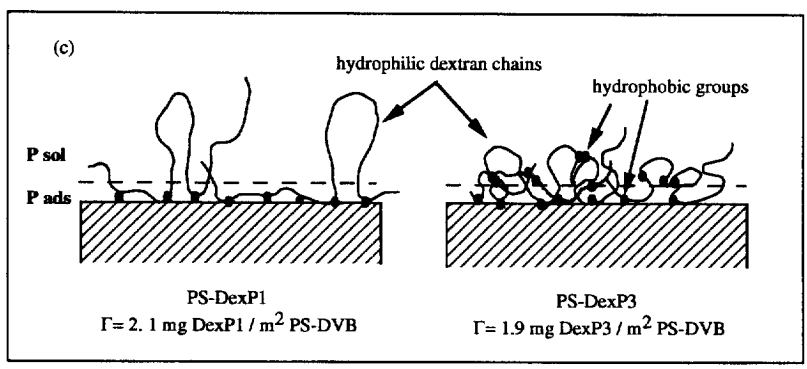


Fig. 3. (a) Evolution with temperature of the ratio of the two populations of the spin labels (P_{sol}/P_{ads} = loops and tails extending into the solution/adsorbed trains) for three samples of hydrophobically modified dextrans adsorbed onto PS–DVB particles (○, DexP1 = 0.05; ◇, DexP2 = 0.33 and □, DexP3 = 0.56 mmol phenoxy groups/g dextran). Amount of adsorbed dexP: Γ = 1.9–2.1 mg/m^2 PS–DVB. (b) Evolution with temperature of the rotational correlation time, τ_c, of the spin labels for loops and tails in the three samples, as defined in (a). The higher the τ_c value, the lower the dextran chains' mobility. (c) Schematic representation of the adsorbed DexP layer at the PS–DVB interface.

The popularity of mixed polymer networks for protein separation purposes has diminished over the last few years. These materials have been progressively replaced by copolymer networks, surface-bonded stationary phases and pore-matrix composites.

A second class is represented by superficially porous microspheres. These media are typically formed by coating microspheres (core particles, average diameter 5–10 μm) with submicronic particles (wall particles) using spray drying or dry impact blending methods. This is intended to develop microparticles with higher surface areas and binding capacities than those of micropellicular (non-

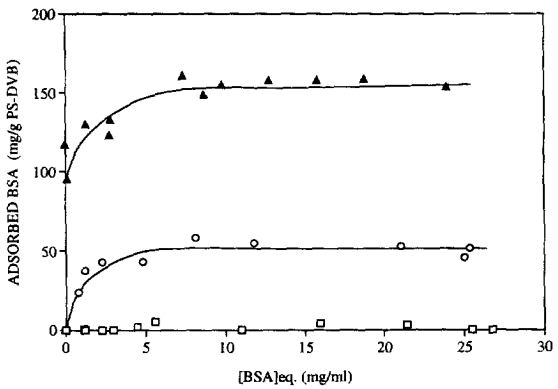


Fig. 4. Adsorption isotherms of BSA on DexP-coated PS–DVB (symbols as defined in Fig. 3) and on uncoated PS–DVB. (▲) Determined in 0.05 *M* phosphate buffer, pH 7.

porous) particles and with improved mass-transfer properties (shorter diffusion path) than totally porous microspheres. This concept was used primarily to prepare sorbents designed for the ion-chromatographic separation of organic and inorganic molecules and involved coating a polystyrene core with charged latex, via electrostatic or hydrophobic interactions [113–115].

In Ref. [116], Kirkland gives details of the preparation of Poroshell particles, where silica microspheres were covered with silica ultramicrospheres. The average pore diameter of Poroshell particles was 300 Å. After surface modification, these particles were used for protein separation in the reversed-phase chromatographic mode and, in terms of efficiency, have been shown to compare favourably with totally porous silica particles having similar diameters and pore size.

Other materials based on polyethylene (PE) have been coated with hydroxyapatite [117] or silica [118]. The characteristics of the silica–PE composite were adjusted by changing the size of both the silica and PE particles. After suitable modification, this material proved to be useful for protein separation, according to a reversed-phase mechanism. Proteins have also been separated on PE–hydroxyapatite using a linear phosphate gradient; in this case, however, irreversible non-specific adsorption with the exposed surface of PE was observed, until saturation occurred.

5. Size-exclusion chromatography

Size exclusion chromatography (SEC) is a separation technique that depends on the hydrodynamic size of proteins, with regard to the pore-size of the support. SEC is frequently used for the fractionation of biomolecules as well as being a useful tool for protein analysis.

5.1. Resolution

In SEC, the calibration curve, i.e. plot of molecular mass versus distribution coefficient, is related to the pore size distribution. Deviations from linearity reflect non-specific interactions, unusual shapes of molecule or can be associated with a multimodal pore size distribution. Selectivity may be improved by decreasing the pore size distribution. The greater the degree of homogeneity in pore diameter, the lower the slope of the calibration curve and the higher the selectivity. The mean pore size should exceed the hydrodynamic diameter of proteins, in order to reduce mass-transfer effects and avoid band broadening. However, increasing the pore size decreases the selectivity and a compromise should be adopted. Poor selectivity can be compensated for by using smaller particles, which provide higher efficiency.

5.2. Loading capacity

The loading capacity of SEC matrices is related to the pore volume and to the pore volume distribution. It is generally low, compared to other chromatographic materials. Due to the low diffusivity of proteins, the separation should be carried out at relatively low linear velocities (50–200 cm/h). Smaller particles (diameter <20 μm) are used for bioanalytical applications; for preparative purposes, the mean particle diameter ranges between 20 and 300 μm.

5.3. Surface chemistry

Ideally, a SEC material should be highly hydrophilic. However, in several cases, non-size-exclusion effects are observed, due to hydrophobic or attractive/repulsive coulombic interactions with the par-

ticle's surface. This drawback is usually overcome by adding salts (0.05–0.2 *M*) or organic modifiers to the mobile phase.

In spite of their low pressure stability, polysaccharides and some polyacrylamide derivatives remain attractive media, due to the absence of non-specific interactions with proteins. Soft gels, such as cross-linked dextran or polyacrylamide, are characterized by an homogeneous flexible network, forming pores of nearly uniform size. This explains why their selectivity is so high. Macroreticular gels, based on agarose, are more rigid but suffer from lower selectivity. In order to circumvent this drawback, materials based on macroreticular agarose, entrapping a soft gel, have been prepared. Superdex (Pharmacia Biotech, Uppsala, Sweden) consists of cross-linked agarose beads whose pores are filled with covalently bound dextran [41]. This material allows high flow-rates (up to 300 cm/h) to be used, and possesses a selectivity comparable to that of Sephadex gels. Moreover, it can be used for analytical as well as for preparative protein separations.

For HPLC applications, preferred media are silica or synthetic organic polymers. Small macroporous silica particles, with relatively uniform pore size, are currently available. The pore size distribution in polymer materials such as PS–DVB or polyacrylate derivatives is generally wider, except for samples prepared on a laboratory scale. Thus, synthetic polymer supports generally have lower selectivity than silica materials [70]. As SEC applications require highly hydrophilic and ion-free surfaces, numerous attempts have been made to modify these materials; it should, however, be noted that polyacrylate-based materials, such as hydroxyethylmethacrylate [70] or oligo(ethylene glycol)dimethacrylate polymers [10], show sufficient hydrophilicity to be used in SEC without any modifications.

Hydrophobic proteins (albumin, lysozyme) or basic proteins (α-chymotrypsinogen A, lysozyme) are commonly used to probe residual hydrophobic or silanolate groups, respectively. Silica and polymeric surfaces can be deactivated by the chemical binding of small hydrophilic molecules, such as glycerol or glucose derivatives [23,67,119–121]. In some cases however, they still exhibit non-specific interactions with proteins and may degrade with time [120,121].

Therefore, multilayer coatings with hydrophilic polymers, including dextran derivatives [84,107], poly(vinyl alcohol) [106], polyether [83], polyglycerol [104] or polyvinylpyrrolidone [87], are usually preferred rather than a monolayer.

The efficiency of hydrophilisation depends strongly on the coating method and on polymer–surface interactions, as described above. Provided that starting base particles with suitable structural characteristics are available, highly resolutive SEC media can be prepared by depositing a thin protecting layer on their surface. A decrease in the thickness and of the permeability of the adsorbed layer can be achieved by a combination of multipoint attachment and dense cross-linking.

Polymer chains that do not interact strongly with the surface, i.e. either one-end grafted polymers, such as "tentacle" materials, pore matrix composites or, to some extent, "fimbriated"-type materials with limited attachment points, provide a less efficient shielding of the core surface than polymers adsorbed via numerous contact points. In addition, the pore size of the starting base materials should be large enough to accommodate proteins after modification. Pore matrix composites based on particles whose pores are filled with dextran have been shown to possess good selectivities in the SEC of proteins [41,42,112]. A new tentacle-type material designed for SEC applications, Fractogel EMD BioSec, has been commercialised recently (Merck, Darmstadt, Germany). This material has tentacles protruding into large pores, forming a porous dynamic network and, thus, could also be regarded as a pore-filled composite. Pore composite materials seem to be quite promising for SEC applications since they combine a rigid structure with a flexible porous network, providing high selectivities and flow-rates.

6. Interactive chromatography

The operating mechanism of interactive chromatography is governed by the reversible binding of proteins to a matrix containing active sites. The theory and practice of the different interactive chromatographic modes are detailed in Refs. [122–124].

For laboratory applications, non-porous particles of small diameter (<5 μm) as well as porous

microparticulate packings (5–10 μm) are employed. For preparative separation purposes, larger particles are preferred, to keep back-pressure at an acceptable level when high flow-rates are required. Irrespective of considerations to do with the ligand itself, the matrix should possess properties similar to those of SEC materials, i.e. it should be inert, to minimize secondary interactions with proteins, and it should be physically and chemically stable. In addition, an ideal support for interactive chromatography applications should possess the following characteristics; contain reactive groups to allow the introduction of ligands and retain porosity in organic solvents in which most activation reactions are carried out.

Numerous packing materials primarily designed for SEC applications are used in interactive modes, after appropriate derivatization. However, materials for interactive chromatography can also be prepared by adsorption onto base particles of polymers bearing pre-introduced functionalities.

Compared to SEC materials, noticeable differences are, however, encountered in factors that govern the choice of a packing material. Special attention must be paid to pore size characteristics and, at a molecular level, to the conformation of the bonded stationary phase.

6.1. Pore size

SEC is based on solute diffusion into the pore matrix and the choice of pore size is governed by the hydrodynamic radii of the proteins to be separated; separation of molecules smaller than 10^6 Da are generally carried out on 300 Å supports and increasing the pore size above certain limits leads to decreased selectivity. In interactive chromatography, it is necessary to use supports with pores large enough to allow the non-restricted access of proteins. Before an attempt is made to select a matrix, one should also keep in mind that binding capacity can decrease dramatically when the size of the protein being isolated increases, depending on pore size distribution. Due to these considerations, proteins smaller than 10^5 molecular mass units are usually separated on supports with a pore diameter of 300–500 Å while a pore diameter of 1000 Å is used for larger proteins. In affinity chromatography, the ligand size should also be taken into account. Enhanced diffusive or convective transport is preferred to classical diffusive transport, especially for fast separations. In this respect, very large pore particles ($\gg$ 1000 Å) as well as non-porous micropellicular materials provide unhindered mass-transport, improving efficiency and the speed of separation. However, such materials have low surface areas and consequently exhibit low binding capacities. Thus, a compromise must be found between high capacity and high diffusivity. When using large pore materials, the drawback of capacity loss can be overcome by filling the pores with a soft gel or by surface derivatization according to the "fimbriated" or "tentacle" technologies.

6.2. Conformational aspects

Another important parameter that plays a determining role in chromatographic performance is the mobility of polymers bearing the chromatographic function. In ion-exchange, hydrophobic and, in some cases, in affinity chromatography, the ligand interacts mostly with the outer surface of proteins, under non-denaturing conditions. As a result, in classical media, functionalities are often directly linked to the support. In some cases, short spacer arms result from the immobilization procedure; longer spacers are mostly used in affinity chromatography, to increase the steric availability of small ligands interacting deeply inside the protein. However, it is now well established that the higher the mobility of polymer bearing the ligand, the higher the selectivity and, to some extent, the capacity, since flexible polymers are capable (i) fitting the shape of a protein without causing distortion or destruction of its structure and (ii) providing an increase in the accessible surface area. In this respect, stationary phases with polymer chains extending far into the solution should be preferred to thin, tightly cross-linked and rigid stationary phases.

6.3. Ion-exchange chromatography

Ion-exchange chromatography (IEC) is a powerful technique that is commonly used for the separation of proteins, based on their charge characteristics. A large majority of purification procedures include an ion-exchange step.

Typically, ion exchangers consist of a matrix substituted with amines as the anion-exchanger, and carboxylic acids as the cation-exchanger. All materials have binding properties that are limited by the pK_a of the acid or base. Therefore, strong acids (sulfonate, sulphate, phosphate) can be used at lower pH values than weak acids (carboxylic acids), while strong bases (quaternary ammonium) can be run at higher pH values than weak bases (diethylaminoethyl, polyethyleneimine).

Besides density and the chemical nature of the functional groups, the most important advances in IEC concern improvements in the structural characteristics of materials, in order to achieve high resolution, speed and capacity. Interesting comparative studies of ion-exchange chromatography separations have been reported recently by Horvath et al. [125]. These studies have been carried out on various ion-exchangers, differing from each other in pore structure. It was found that gel in shell HyperD (Biosepra, France) and perfusive Poros particles (Perseptive Biosystems, MA, USA) have a higher efficiency than the traditional porous matrix, monoQ/S (Pharmacia Biotech). By increasing the flow-rate, the efficiency and capacity of both HyperD and monoQ progressively decrease, while the properties of Poros remain unchanged up to 600 cm/h. However, even at high flow-rates, the dynamic capacity of HyperD remains higher than that of the other materials. Moreover, the authors reported higher resolving power of the conventional material at low protein loading, whereas at high loading, HyperD compares favourably with other materials.

Another comparative study reflecting the influence of polymer mobility on chromatographic separations has been published by Ivanov and Zubov [85]. They compared anion-exchanger bonded phases, composed of multivalently adsorbed polymer layers and reported that poly(N-diethylaminoethylacrylamide) layer, bonded to γ-aminopropylsilica via (i) chemical adsorption of poly(*p*-nitrophenyl acrylate) and (ii) coupling to 2-diethylaminoethylamine, exhibits improved selectivity in protein separation than thin cross-linked diethylaminoethyl (DEAE) dextran or polyethyleneimine (PEI) layers. To explain these differences, the authors expected longer loops and tails in the polyacrylamide layer than in PEI or DEAE dextran layers, due to differences in the coating technology. However, they reported lower binding capacity than that expected for "fimbriated"- or "tentacle"-like materials. Whether loops and tails are long and flexible enough to increase ligand availability should be carefully examined using techniques such as EPR or NMR.

The comparison of two commercial ion-exchange supports can also provide some light on the role of polymer conformation: Toyopearl and Fractogel EMD (Merck) have been developed from the same base polyacrylate material. Both supports are functionalized in order to introduce ion-exchange functionalities. The main difference lies in the presence of tentacles at the surface of Fractogel EMD. Fractogel EMD provides more resolutive separations and exhibits higher capacity than diffusive Toyopearl; as an example, the binding capacity of DEAE Toyopearl 650 (1000 Å pore diameter) is 25–35 mg protein/ml, while the binding capacity of Fractogel EMD DEAE 650 was found to be 70–140 mg protein/ml (manufacturers' data). The mechanism of protein adsorption on pellicular- and tentacle-type ion-exchangers has been investigated by Janzen et al. [126]; these authors studied different modes of adsorption and showed that the binding capacity of tentacle sorbents largely exceeds that corresponding to a BSA monolayer. However, they expected higher negative binding cooperativity on the tentacle-type material, due to deformation of the layer in the presence of a first adsorbed protein, causing decreased affinity for a second, neighbouring protein molecule.

6.4. Hydrophobic interaction chromatography

In HIC, proteins separate by their surface hydrophobicity. Salts in buffer (1–3 *M*) promote hydrophobic binding, while a decreasing salt gradient is used to elute samples from the column.

As mentioned in previous sections, the capacity and specificity of separations depend on the physicochemical characteristics of the matrix, as well as on the amount of accessible ligands. The influence of different parameters on HIC separations, including physico-chemical characteristics of sorbents as well as elution conditions, continue to be studied extensively [30,127,128]. Earliest supports were prepared by alkylation of polysaccharide materials. With im-

provement of their flow properties, they are still useful and continue to be developed extensively [129–133].

Later efforts have also been directed towards the use of more rigid materials. Compared to reversed-phase matrices, there is still a need to cover the core surface to provide an inert and hydrophilic layer. Next, moderately hydrophobic surfaces are produced by grafting alkyl or aryl groups onto the hydrophilic layer [40,71,134]. Relatively low ligand concentrations (100–1000 μmol/ml) are required, in order to avoid disruption of the protein structure. This contrasts with reversed-phase systems where proteins are generally eluted in a denatured state, due to the high ligand density.

To satisfy different selectivities, various matrices are provided with a large choice of ligands and substitution levels. Usually, functional groups are attached to the hydrophilic matrix by reacting epichlorohydrin, butanediol diglycidyl ether or monoglycidyl ethers. Monofunctional epoxides allow the introduction of ligand without negative side effects. When using difunctional reagents, additional cross-linking may occur within the hydrophilic layer. The most commonly used ligands are alkyl chains, in the range C_2 to C_8 (methyl, propyl, butyl, hydropropyl, neopentyl...) and phenyl groups. The choice of ligand, i.e. more or less hydrophobic, is directed by the hydrophobic character of the proteins to be separated. Generally speaking, the stronger the hydrophobicity of the protein, the weaker the hydrophobicity of the ligand to be used.

Alternatively, sorbents for HIC applications can be prepared by direct immobilization of polymers having mild hydrophobic properties onto the surface of particles. This concept was first introduced by Ling and Mattiasson [135], who separated proteins on poly(ethylene glycol)- or poly(vinyl alcohol)-bonded Sepharose. A number of protein separations by mean of HIC were demonstrated on polyether, PVP or PVA-bonded phases. In particular, Kurganov et al. [87] reported that PVP-coated silica can perform as a SEC media at low ionic strength (0.2 *M* NaCl), whereas it retains most proteins at 2.8 *M* $(NH_4)_2SO_4$. The proteins are eluted from the column by decreasing the ionic strength, with only minor differences compared to conventional HIC supports. In our laboratory, we observed similar effects with PVA-coated polystyrene. We showed that the physical adsorption of PVA onto macroporous polystyrene particles, followed by cross-linking, provides a suitable material for the SEC of proteins [106]. However, hydrophobic interactions with BSA have been evidenced by increasing the ionic strength and the protein is totally retained at 2 *M* NaCl. Other stationary phases for HIC chromatography have been prepared in our group by covalent immobilization of various polyether derivatives [poly(ethylene glycol) (PEG), copolymers of PEG–PPG] on Sepharose and these have been used for the separation of standard or complex protein mixtures [136]. Furthermore, hydroxyethylmethacrylate beads show HIC capability in their native form, as studied by Kleinmann et al. [137].

A totally different approach in preparing HIC sorbents consists of modifying reversed-phase support properties. Recently, a reversed-phase column ($Si–C_{18}$) was dynamically coated with a non-ionic polyoxyethylene surfactant without any covalent binding. Pure aqueous mobile phases, with no surfactant added, have been used to elute the proteins. The separation of a protein mixture has been shown to be reproducible over 40 runs and an efficient one-step purification of a fungal aspartic proteinase from a crude sample was obtained [138].

6.5. Affinity chromatography

The development of a separation method may include several steps (five or more) from the crude sample to the final purified protein. Since AC exploits selective interactions between a ligand and a target molecule, it can therefore be used to simplify purification schemes and one-step purification procedures are commonly obtained. On the other hand, affinity chromatography is a powerful technique for separating and concentrating minor components of interest from a complex mixture.

As in the case of supports used in other interactive modes of chromatography, stable hydrophilic macroporous or non-porous matrices can be used. The structural parameters to consider still remain particle-size, pore-size distribution, available specific area and polymer flexibility, since high ligand accessibility may be achieved through the use of mobile polymeric arms.

However, the kinetics of adsorption in affinity chromatography (AC) are slower than in IEC or HIC and the contribution of polymer chain conformation and mobility at the interface is less critical in the mechanism of separation, in terms of mass-transfer resistance.

Irrespective of structural considerations, stationary phases are often tailor-made for the purification of a given protein and each separation process must be optimized. Ligands are classified into two main categories; monospecific and group-specific. Generally, monospecific ligands bind more strongly to proteins than group-specific ligands, and association constants in the range 10^6–10^8 M^{-1} are generally reported. Interactions characterized by smaller constants (10^3–10^5 M^{-1}) may cause protein retardation rather than the formation of a stable complex. Above 10^9 M^{-1}, drastic conditions are required to elute the protein.

While biological ligands (enzymes, receptors, antibodies, protein A, lectin, nucleic acids) continue to be extensively used [40,68,78,104,108,139–151], their application is hampered by their high cost and/or their relative instability. In order to overcome these drawbacks, continuous efforts are oriented towards the use of more robust ligands, capable of replacing natural ligands.

Among the synthetic ligands, low-molecular-mass compounds, including dye, chelated metal, thiophilic ligands and boronic acids derivatives, have become extremely popular [19,40,42,43,78,105,139,152–157]. As synthetic ligands are often group-specific ligands, they are able to interact with different proteins simultaneously and thus exhibit moderate specificity. For this reason, improving the selectivity of separations is of significant importance. This can be achieved by using a competitive biological ligand, a competitive displacer or by designing new ligands that are tailor-made for the target protein.

Galaev et al. [158] have developed a new approach, named "shielding affinity chromatography". To accomplish this, they adsorbed, on dye–Sepharose complexes, different polymers including PVP or PVA, which were capable of interacting with the dye molecule at an intermediate level between very specific and less specific interactions. Such a system significantly reduces non-specific interactions and has been successfully applied to the purification of phosphofructokinase, lactate dehydrogenase and secondary alcohol dehydrogenase [159,160]. In addition, they reported on the utility of polyvinylcaprolactone (PVCL) for the polymer shielding of blue Sepharose [161]. PVCL is a thermosensitive polymer that demonstrates good solubility in water at low temperature, i.e. below LCST (38°C) but separates from solution when the temperature is raised above LCST, due to a coil–globule transition [162]. Above LCST, PVCL molecules shrink and the availability of ligands increases. By decreasing the temperature, PVCL extends in solution, interacts more strongly with the dye molecules and displaces bound proteins. A PVCL–blue Sepharose system was tested for the separation of lactate dehydrogenase; the purification factor was seventeen and protein recovery was 90%.

Another route to produce stable affinity materials exploits the particular affinity of some polymers for proteins. As recent examples, one may mention the affinity of cross-linked amylose (a 1–4 glycosidic bond) for haemoglobin [60,163] or the selective removal of endotoxins from protein solutions using amino poly(γ-methyl L-glutamate) particles [164] or cross-linked N,N′-dimethylaminopropyl–acrylamide particles [165]. Copper ions have been proved recently to complex with cross-linked dextran–polyimine stationary phase and preliminary results demonstrating the utility of such a system in ligand-exchange chromatography have been reported, using BSA as a test molecule [166]. In another study, polyphosphates were adsorbed onto porous zirconia particles; since polyphosphates are capable of forming complexes with some proteins and nucleic acids, this property has been exploited for the purification of RNase A, DNase I and alkaline phosphatase [167].

It is well known that the reagent used as a cross-linker or for ligand immobilization can induce non-specific interactions, depending on their ionic and/or hydrophobic characteristics. Recently, the thiophilic nature of divinylsulfone cross-linked agarose was reported by Noel et al. [64] and IgG binding studies suggest that this gel could compare favourably with the commercial thiophilic Tgel (ligand divinylsulfone–2 mercaptoethanol).

In a few cases, the physico-chemical properties of stationary phases have been used for the physical attachment of ligands. Such an approach may pro-

vide easily regenerable materials, at a lower cost than that of conventional materials, since no chemistry is required to immobilize the ligand. For instance, Procion-Brown H-A, physically adsorbed on a PVP column, was used to purify egg white lysozyme. A 37-fold purification of the enzyme was achieved, with recovery of 80% of the activity [168]. In a similar way, concanavalin A was immobilized onto zirconia, via Lewis acid–base interactions; additionally, concanavalin A was cross-linked with glutaraldehyde to increase the stability of the column. This material was easily regenerated, by removing the protein covering the surface and reloading the column. The authors showed that the behaviour of adsorbed concanavalin was similar to that of concanavalin that was covalently immobilized on silica, using *p*-nitrophenyl sugars as probes [169].

In the design of AC supports, a new concept introduced a few years ago consists of creating an artificial binding site for protein in the stationary phase, by molecular imprinting techniques. As shown in Fig. 5, a functional monomer interacting with a print molecule is allowed to polymerize or to react with the derivatized surface. In the following step, the print molecule is removed from the matrix. Then the obtained material is capable of being recognised specifically and of rebinding the print molecule in the presence of other molecules.

Within the past few years, a considerable amount of work has been devoted to the use of molecularly imprinted polymer for enantiomeric separations. Recently, Kempe and Mosbach [98] described the preparation of silica surface imprinting of proteins. Silica was first derivatized with methacrylate groups and then allowed to react with a metal binding monomer, N-(4-vinyl)benzyl iminodiacetic acid in the presence of RNase A and copper ions. Similarly, a support was prepared using BSA as a print molecule. After removing proteins, the RNase A-imprinted phase was shown to bind RNase with a higher affinity than the BSA-imprinted support.

Another interesting example has been provided by Liao et al. [170]. Acrylamide and N,N′-methylenebisacrylamide were copolymerized in the presence of haemoglobin, cytochrome *c* or transferrin. The entrapped protein was then removed by washing the column. The method slightly differs from the preceding one as no specific complexes are formed between

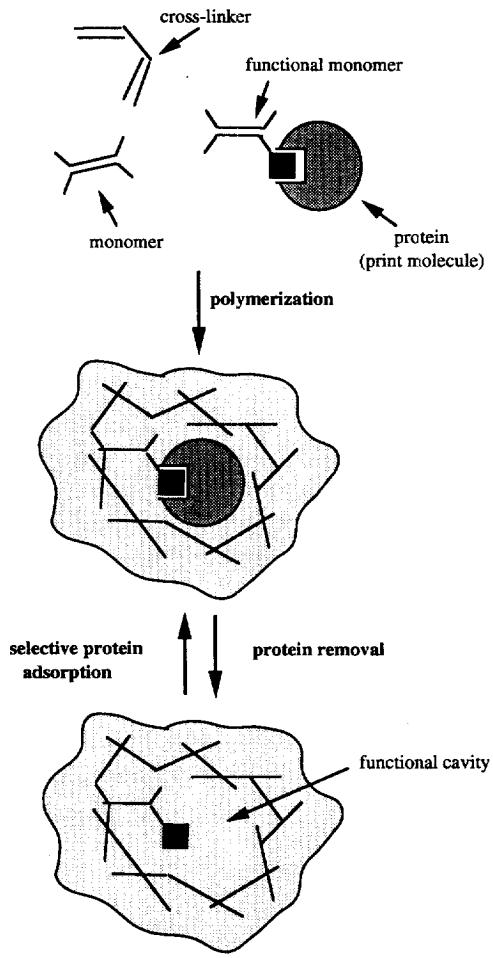


Fig. 5. Schematic representation of molecular imprinting (adapted from Ref. [98]).

the monomer and the print protein. However, recognition sites are formed and, for instance, cytochrome *c* adsorbed only on the material synthesized in the presence of cytochrome *c*. According to the authors, the mechanism of selectivity requires clarification since no ligands were introduced during the preparation of supports.

7. Restricted access packing materials

These packings have been developed for the direct analysis and separation of protein solutions containing small analytes such as drugs and are char-

acterized by mixed hydrophilic–hydrophobic properties (Fig. 6). Eluants generally used are phosphate buffers, containing small amounts of organic solvents such as THF or acetonitrile. Proteins are excluded from the columns while hydrophobic drugs are eluted according to a reversed-phase mechanism.

A first class of restricted access media consists of particles with a hydrophobic layer, surrounded by a hydrophilic layer that is permeable to low-molecular-mass compounds only. Restricted access packing materials of this type are prepared following two main approaches.

Mixed functional phases are obtained by multi-step procedures. Kanda et al. [91,92] reported on silica modifications including (i) surface passivation with a silicone layer, (ii) introduction of hydrophobic (methyl, octyl, styrenic) groups and (iii) introduction of hydrophilic (oligoglyceryl or polyoxyethylene) groups. Retention of drug molecules as well as protein recovery were increased by replacing the oligoglyceryl groups by polyoxyethylene groups. However, protein recovery decreased on increasing the hydrophobicity of the internal layer [91,92].

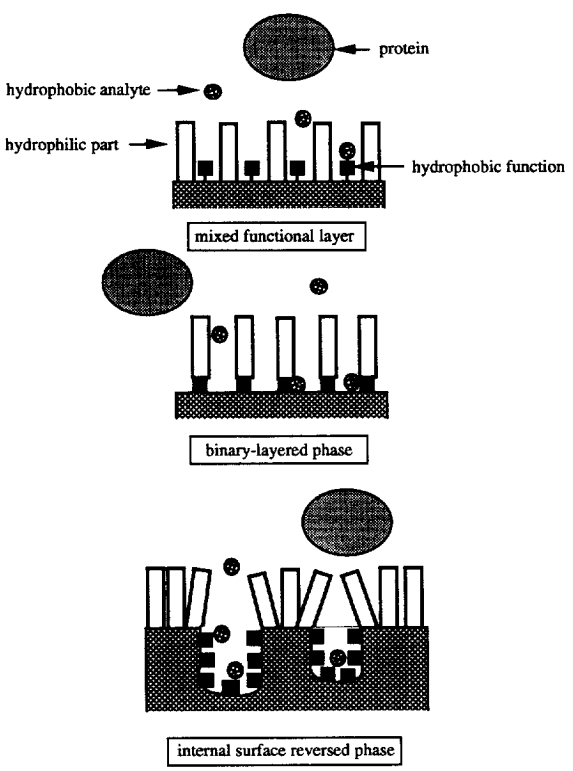


Fig. 6. Schematic structure of restricted access packing materials (adapted from Ref. [171]).

Binary layered phases are obtained by binding a ligand containing both hydrophilic and hydrophobic parts in such a way that the hydrophobic segment is located on the inside of the attached layer. They can also be obtained by immobilization of hydrophilic ligands using hydrophobic coupling reagents. Nimura et al. [171] reported on the preparation of 3-(2,3)hydroxypropylsilylsilica and efficiently separated drugs added to a HSA solution, with 100% protein recovery.

A second class of restricted access media, named "internal surface reversed-phase" consists of porous materials in which small pores (<20 Å) are made hydrophobic while larger pores are hydrophilic. This can be achieved directly during particle polymerization by choosing appropriate monomers and polymerization conditions, or by pore size-specific functionalization.

Hosoya et al. [172] showed that particles based on poly(glycerolmethacrylate–glyceroldimethacrylate) exhibit sufficient hydrophobicity for direct use as drug separation media. Moreover, they claimed that all materials prepared by radical polymerization of a hydrophilic monomer in the presence of a cross-linking monomer present hydrophobic micropores comprising a cross-linked structure of hydrophobic carbon chains and can, therefore, be used for protein–drug separations.

Fréchet's group has been very active in the field of pore size-specific functionalization. For instance, they prepared particles based on poly(glycidylmethacrylate–co-ethylenedimethacrylate); glycidyl groups were then specifically hydrolysed in large pores by using a polymeric acid, while smaller pores were further derivatized with hydrophobic amines or alcohols [71,72,173]. In another study, they showed that modification of polystyrene particles prepared in the presence of a hydrophobic porogen, with a hydrophilic monomer added during the final polymerization step, occurs specifically onto the external surface of particles [95]. Similarly, a temperature-responsive polymer based on poly(N-isopropylacrylamide) was deposited onto methacrylate beads prepared either with cyclohexanol or toluene as the porogen. Since PNIPAM is soluble in hexanol

and insoluble in toluene, changing the porogen results either in an internally or an externally modified surface, respectively. The packing with an externally modified surface was capable of removing drugs that coexisted with proteins such as BSA, but the percentage of BSA recovery drastically decreased on raising the temperature above the LCST of PNIPAM [97].

8. Conclusions

With the introduction of new base materials, improvements in the structural characteristics of particles and an increase in the variety of organic polymers, rational procedures have been developed in order to design composite media with improved physico-chemical stability, selectivity and efficiency.

In polymer-coated media, chromatographic performances are strongly related to the chemical nature of the adsorbed polymer, as well as to its conformation in the deposited layer. Thus, a thin and densely packed layer can efficiently prevent non-specific protein adsorption, whereas flexible polymer chains can enhance site accessibility and decrease mass-transfer resistance, provided that, in porous media, modification does not restrict pore access.

During the past few years, several novel packing materials have emerged, to satisfy the need for high throughput separations permitted by improvement of mass-transfer. These new media include perfusive materials, pore-filled composites and tentacle-like sorbents.

Future trends in surface design will probably be devoted to the preparation of tailor-made materials for specific applications, using not only synthetic and low cost ligands, but also new procedures such as imprinting techniques or pore-size functionalization. Moreover, the use of polymers with particular features, i.e. capable of promoting specific interactions with proteins under defined pH, ionic strength or temperature conditions should develop rapidly.

References

[1] R. Arshady, J. Chromatogr. 586 (1991) 181.
[2] R. Arshady, J. Chromatogr. 586 (1991) 199.
[3] J.W. Vanderhoff, M.S. El-Aasser, F.J. Micale, E.D. Sudol, C.M. Tseng, A. Silwanowicz, H.R. Sheu, D.M. Kornfeld, Polym. Mater. Sci. Eng. 54 (1986) 587.
[4] J.H. Kim, E.D. Sudol, M.S. El-Aasser, J.W. Vanderhoff, D.M. Kornfeld, Chem. Eng. Sci. 43 (1988) 2025.
[5] T. Ellingsen, O. Aune, J. Ugelstad, S. Hagen, J. Chromatogr. 535 (1990) 147.
[6] T. Ellingsen, O. Aune, A. Berge, L. Kilaas, R. Schmid, P. Stenstad, J. Ugelstad, S. Hagen, E. Weng, L. Johansen, Macromol. Chem., Macromol. Symp. 70/71 (1993) 315.
[7] K. Hosoya, J.M.J. Fréchet, J. Liq. Chromatogr. 16 (1993) 353.
[8] V. Smigol, F. Svec, K. Hosoya, Q. Wang, J.M.J. Fréchet, Angew. Macromol. Chem. 195 (1992) 151.
[9] V. Smigol, F. Svec, J.M.J. Fréchet, J. Liq. Chromatogr. 17 (1994) 259.
[10] K. Ogino, H. Sato, Y. Aihara, H. Suzuki, S. Moriguchi, J. Chromatogr. A 699 (1995) 167.
[11] K. Ogino, H. Sato, K. Tsuchiya, H. Suzuki, S. Moriguchi, J. Chromatogr. A 699 (1995) 59.
[12] Q.C. Wang, K. Hosoya, F. Svec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 1232.
[13] Q.C. Wang, F. Svec, J.M.J. Fréchet, Polym. Bull. 28 (1992) 569.
[14] C.M. Cheng, F.J. Micale, J.W. Vanderhoff, M.S. El-Aasser, J. Polym. Sci., Part A: Polym. Chem. 30 (1992) 235.
[15] C.M. Cheng, J.W. Vanderhoff, M.S. El-Aasser, J. Polym, J. Polym. Sci., Part A: Polym. Chem. 30 (1992) 245.
[16] D.K. Roper, E.N. Lightfoot, J. Chromatogr. A 702 (1995) 3.
[17] S. Hjerten, Y.M. Li, J.L. Liao, K. Nakazato, G. Pettersson, Nature 356 (1992) 810.
[18] F. Svec, J.M.J. Fréchet, Anal. Chem. 64 (1992) 820.
[19] J.M.J. Fréchet, Macromol. Chem., Macromol. Symp. 70/71 (1993) 289.
[20] K. Nakazato, J. Mohammad, S. Hjerten, Chromatographia 39 (1994) 655.
[21] Q.C. Wang, F. Svec, J.M.J. Fréchet, Anal. Chem. 65 (1993) 2243.
[22] F. Svec, J.M.J. Fréchet, J. Chromatogr. A 702 (1995) 89.
[23] Q.C. Wang, F. Svec, J.M.J. Fréchet, Anal. Chem. 67 (1995) 670.
[24] J.L. Liao, Y.M. Li, S. Hjerten, Anal. Biochem. 234 (1996) 27.
[25] J.D. Andrade, V. Hlady, Adv. Polym. Sci. 79 (1986) 1.
[26] W. Norde, Cells Materials 5 (1995) 97.
[27] J.L. Brash and T.A. Horbett, in T.A. Horbett and J.L. Brash (Editors), Proteins at Interfaces II: Fundamentals and Applications, ACS Symposium Series 602; American Chemical Society: Washington, DC, 1995, Ch 1, p. 1.
[28] C. Schöneich, A.F.R. Hühmer, S.R. Rabel, J.F. Stobaugh, S.D.S. Jois, C.K. Larive, T.J. Siahaan, T.C. Squier, D.J. Bigelow, T.D. Williams, Anal. Chem. 67 (1995) 155R.
[29] Q.M. Mao, R. Stockmann, I.G. Prince, M.T.W. Hearn, J. Chromatogr. 646 (1993) 67.
[30] A. Tongta, A.I. Liapis, D.J. Siehr, J. Chromatogr. A 686 (1994) 21.
[31] A. Liapis, Y. Xu, O.K. Crosser, A. Tongta, J. Chromatogr. A 702 (1995) 45.

[32] A. Jungbauer, J. Chromatogr. 639 (1993) 3.
[33] S.M. Cramer, G. Jayaraman, Curr. Opin. Biotechnol. 4 (1993) 217.
[34] A. Jungbauer, E. Boschetti, J. Chromatogr. B 662 (1994) 143.
[35] E. Boschetti, J. Chromatogr. A 658 (1994) 207.
[36] G. Sofer, J. Chromatogr. A 707 (1995) 23.
[37] E.C. Nice, Biopolymers 40 (1996) 319.
[38] H. Chen, Cs. Horvath, J. Chromatogr. A 705 (1995) 3.
[39] N.B. Afeyan, N.F. Gordon, I. Mazsaroff, L. Varady, S.P. Fulton, Y.B. Yang, F.E. Regnier, J. Chromatogr. 519 (1990) 1.
[40] N.B. Afeyan, S.P. Fulton, F.E. Regnier, J. Chromatogr. 544 (1991) 267.
[41] L. Kagedal, B. Engström, H. Ellegren, A.K. Lieber, H. Lundström, A. Sköld, M. Schenning, J. Chromatogr. 537 (1991) 17.
[42] D. Mislovicova, M. Petro, D. Berek, J. Chromatogr. 646 (1993) 411.
[43] D. Mislovicova, M. Kovacova, M. Petro, P. Bozek, J. Liq. Chromatogr. 18 (1995) 3061.
[44] E. Boschetti, L. Guerrier, P. Girot, J. Horvath, J. Chromatogr. B 664 (1995) 225.
[45] K.K. Unger, K.D. Lork and H.J. Wirth, in M.T. Hearn (Editor), HPLC of Proteins, Peptides and Polynucleotides, VCH, New York, 1991, p. 59.
[46] I. Novak, D. Berek, J. Chromatogr. A 665 (1994) 33.
[47] S.M. Fields, Anal. Chem. 68 (1996) 2709.
[48] G.B. Cox, J. Chromatogr. A 656 (1993) 353.
[49] J.J. Kirkland, Am. Lab. (1994) 28K.
[50] J.J. Kirkland, C.H. Dilks, J.J. DeStefano, J. Chromatogr. 635 (1993) 19.
[51] J. Nawrocki, M.P. Rigney, A. McCormick, P.W. Carr, J. Chromatogr. A 657 (1993) 229.
[52] U. Trüdinger, G. Müller, K.K. Unger, J. Chromatogr. 535 (1990) 111.
[53] C.J. Dunlap, P.W. Carr, A.V. McCormick, Chromatographia 42 (1996) 273.
[54] L. Sun, M.J. Annen, F. Lorenazano-Porras, P.W. Carr, A.V. McCormick, J. Colloid Interface Sci. 163 (1994) 464.
[55] A. Kurganov, U. Trüdinger, T. Isaeva, K. Unger, Chromatographia 42 (1996) 217.
[56] J.A. Blackwell, P.W. Carr, J. Chromatogr. 596 (1992) 27.
[57] S. Inoue, N. Ohtaki, J. Chromatogr. 645 (1993) 57.
[58] S. Inoue, N. Ohtaki, J. Chromatogr. 679 (1994) 247.
[59] M.J. Gorbunoff, Anal. Biochem. 136 (1984) 440.
[60] W.J. Jacques, M.A. Mateescu, J. Mol. Recognit. 8 (1995) 106.
[61] V.P. Torchilin, M.I. Papisov, J. Liposome Res. 4 (1994) 725.
[62] H.F. Boeden, K. Pommerening, M. Becker, C. Rupprich, M. Holtzhauer, F. Loth, R. Muller, D. Bertram, J. Chromatogr. 552 (1991) 389.
[63] P.E. Gustavsson, P.O. Larsson, J. Chromatogr. A 734 (1996) 231.
[64] R.J. Noel, W.T. O'Hare, G. Street, J. Chromatogr. A 734 (1996) 241.
[65] E. Boschetti, J. Biochem. Biophys. Methods 19 (1989) 21.
[66] S. Hjerten, J. Mohammad, K. Nakazato, J. Chromatogr. 646 (1993) 121.
[67] P.R. Johnson, N.J. Stern, P.D. Eitzman, J.K. Rasmussen, D.S. Milbrath, R.M. Gleason, R.E. Hogancamp, J. Chromatogr. A 667 (1994) 1.
[68] G.T. Hermanson, G.R. Mattson, R.I. Krohn, J. Chromatogr. A 691 (1995) 113.
[69] J. Coupek, M. Krivakova, S. Pokorny, J. Polym. Sci.: Polym. Symp. 42 (1973) 185.
[70] J. Coupek, I. Vins, J. Chromatogr. A 658 (1994) 391.
[71] V. Smigol, F. Svec, J.J.M. Fréchet, Anal. Chem. 66 (1994) 2129.
[72] V. Smigol, F. Svec, J.J.M. Fréchet, Anal. Chem. 66 (1994) 4308.
[73] Q.C. Wang, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 669 (1994) 230.
[74] R. Murakami, H. Hachisako, K. Yamada, Y. Motazoto, J. Chromatogr. A 678 (1994) 180.
[75] W. Müller, J. Chromatogr. 510 (1990) 133.
[76] F. Schindler, H. Schmidbaur, Angew. Chem. 79 (1967) 697.
[77] H.J. Wirth, K.O. Eriksson, P. Holt, M. Aguilar, M.T.W. Hearn, J. Chromatogr. 646 (1993) 129.
[78] H.J. Wirth, M.T.W. Hearn, J. Chromatogr. 646 (1993) 143.
[79] H.H. Freiser, K.M. Gooding, J. Chromatogr. 544 (1991) 125.
[80] M. Petro, D. Bereck, Chromatographia 37 (1993) 549.
[81] P.G. Mingalyov, A.Y. Fadeev, J. Chromatogr. A 719 (1996) 291.
[82] A. Kurganov, V. Davankov, K. Unger, J. Chromatogr. 548 (1991) 207.
[83] Y. Frere, P. Gramain, React. Polym. 16 (1991) 137.
[84] M. Petro, P. Gemeiner, D. Bereck, J. Chromatogr. A 665 (1994) 37.
[85] A.E. Ivanov, V. Zubov, J. Chromatogr. A 673 (1994) 159.
[86] A.E. Ivanov, S.V. Belov, V. Zubov, Polym. Sci. 36 (1994) 276.
[87] A. Kurganov, Y. Puchkova, D. Davankov, F. Eisenbeiss, J. Chromatogr. A 663 (1994) 163.
[88] A.D. Kataev, V.V. Saburov, O.A. Reznikova, D.V. Kapustin, V.P. Zubov, J. Chromatogr. A 660 (1994) 131.
[89] A. Kurganov, V. Davankov, T. Isajeva, K. Unger, F. Eisenbeiss, J. Chromatogr. A 660 (1994) 97.
[90] H. Kutsuna, Y. Ohtsu, M. Yamaguchi, J. Chromatogr. 635 (1993) 197.
[91] T. Kanda, H. Kutsuna, Y. Ohtsu, M. Yamaguchi, J. Chromatogr. A 672 (1994) 51.
[92] T. Kanda, O. Shirota, Y. Ohtsu, M. Yamaguchi, J. Chromatogr. A 722 (1996) 115.
[93] Y.B. Yang, K. Harrison, J. Kindsvater, J. Chromatogr. A 723 (1996) 1.
[94] Y.C. Liang, F. Svec, J.M.J. Fréchet, J. Polym. Sci., Part A: Polym. Chem. 33 (1995) 2639.
[95] K. Hosoya, Y. Kishii, K. Kimata, T. Araki, N. Tanaka, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 690 (1995) 21.
[96] M. Gewehr, K. Nakamura, N. Ise, H. Kitano, Macromol. Chem. 193 (1992) 249.
[97] K. Hosoya, K. Kimata, T. Araki, N. Tanaka, J.M.J. Fréchet, Anal. Chem. 67 (1995) 1907.

[98] M. Kempe, K. Mosbach, J. Chromatogr. A 691 (1995) 317.
[99] M. Hanson, B. Eray, K. Unger, A.V. Neimark, J. Schmid, K. Albert, E. Bayer, Chromatographia 35 (1993) 403.
[100] L. Sun, A.V. McCormick, P.W. Carr, J. Chromatogr. A 658 (1994) 465.
[101] L. Sun, P.W. Carr, Anal. Chem 67 (1995) 3717.
[102] M. Hanson, K.K. Unger, C.T. Mant, R.S. Hodges, J. Chromatogr. 599 (1992) 65–77.
[103] J. Renard, C. Vidal-Madjar, B. Sébille, J. Liq. Chromatogr. 15 (1992) 71.
[104] L. Varady, N. Mu, Y.B. Yang, S.E. Cook, N. Afeyan, F.E. Regnier, J. Chromatogr. 631 (1993) 107.
[105] A. Tuncel, A. Denizli, D. Purvis, C.R. Lowe, E. Piskin, J. Chromatogr. 634 (1993) 161.
[106] M. Léonard, C. Fournier, E. Dellacherie, J. Chromatogr. B 664 (1995) 39.
[107] C. Fournier, M. Leonard and E. Dellacherie, Int. J. Biochromatogr., in press.
[108] H. Lakhiari, E. Legendre, D. Muller, J. Jozefonvicz, J. Chromatogr. B 664 (1995) 163.
[109] M.P. Vivarat-Perrin, B. Sebille, C. Vidal-Madjar, J. Chromatogr. 584 (1992) 3.
[110] C.J. Dunlap, P.W. Carr, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 2059.
[111] C. Fournier, Ph.D. Thesis, Institut National Polytechnique de Lorraine, 1996.
[112] M. Petro, D. Berek, I. Novak, React. Polym. 23 (1994) 173.
[113] J.R. Stillian, C.A. Pohl, J. Chromatogr. 499 (1990) 249.
[114] R.F. Strasburg, J.S. Fritz, J.O. Naples, J. Chromatogr. 547 (1991) 11.
[115] R.C. Ludwig, J. Chromatogr. 592 (1992) 101.
[116] J.J. Kirkland, Anal. Chem. 64 (1992) 1239.
[117] F. Honda, H. Honda, M. Koishi, J. Chromatogr. A 696 (1995) 19.
[118] F. Honda, H. Honda, M. Koishi, J. Chromatogr. 609 (1992) 49.
[119] R.E. Huisden, J.C. Kraak, H. Hope, J. Chromatogr. 505 (1990) 289.
[120] H.G. Lee, H.W. Jarrett, J. Chromatogr. A 653 (1993) 130.
[121] R. Ovalle, Anal. Biochem. 229 (1995) 1.
[122] Janson-Ryden (Editor), Protein Purifications, VCH, New York, 1989.
[123] M.T. Hearn (Editor), HPLC of Proteins, Peptides and Polynucleotides, VCH, New York, 1991.
[124] T.T. Ngo (Editor), Molecular Interactions in Bioseparations, Plenum Press, New York, 1993.
[125] J. Horvath, E. Boschetti, L. Guerrier, N. Cooke, J. Chromatogr. A 679 (1994) 11.
[126] R. Janzen, K.K. Unger, W. Müller, M.T.W. Hearn, J. Chromatogr. 522 (1990) 77.
[127] L. Szepesy, G. Rippel, J. Chromatogr. A 668 (1994) 337.
[128] A. Oscarsson, J. Chromatogr. B 666 (1995) 21.
[129] A. Berggrund, I. Drevin, K.G. Knuuttila, J. Wardhammar, B.L. Johansson, Process Biochem. 29 (1994) 445.
[130] G. Rippel, L. Szepesy, J. Chromatogr. A 664 (1994) 27.
[131] A. Serys, J. Liesiene, J. Urbonaviciene, A. Maruska, K. Radzevicius, D. Valatkeviciute, V. Bummels, J. Liq. Chromatogr. 17 (1994) 749.
[132] S. Oscarsson, D. Angula-Tatis, G. Chaga, J. Porath, J. Chromatogr. A 689 (1995) 3.
[133] J.A. Queiroz, F.A.P. Garcia, J.M.S. Cabral, J. Chromatogr. A 734 (1996) 213.
[134] P. Smidl, I. Kleinmann, J. Plicka, V. Svoboda, J. Chromatogr. 523 (1990) 131.
[135] T.G.I. Ling, B. Mattiasson, J. Chromatogr. 254 (1983) 83.
[136] P. Hubert, R. Mathis, E. Dellacherie, J. Chromatogr. 539 (1991) 297.
[137] I. Kleinmann, J. Plicka, P. Smidl, I. Vins, Int. Chromatogr. Lab. 22 (1994) 18.
[138] Y.L.K. Sing, Y. Kroviarski, S. Cochet, D. Dhermy, O. Bertrand, J. Chromatogr. 598 (1992) 181.
[139] D.J. Phillips, B. Bell-Alden, M. Cava, E.R. Grover, W.H. Mandeville, R. Mastico, W. Sawlivich, G. Vella, A. Weston, J. Chromatogr. 536 (1991) 95.
[140] Y. Yang, F.E. Regnier, J. Chromatogr. 544 (1991) 233.
[141] J. Tharakan, F. Highsmith, D. Clark, W. Drohan, J. Chromatogr. 595 (1992) 103.
[142] N. Ubrich, P. Hubert, V. Regnault, E. Dellacherie, C. Rivat, J. Chromatogr. 584 (1992) 17.
[143] K. Isosaki, N. Seno, I. Matsumoto, T. Koyama, S. Moroguchi, J. Chromatogr. 597 (1992) 123.
[144] N. Ubrich, P. Hubert, V. Regnault, E. Dellacherie, C. Rivat, Biotechnol. Blood Prot. 227 (1993) 43.
[145] W. Guo, Z. Shang, Y. Yu, L. Zhou, Biomed. Chromatogr. 8 (1994) 142.
[146] A.A. Farooqui, H.C. Yang, L.A. Horrocks, J. Chromatogr. A 673 (1994) 149.
[147] W.C. Lee, C.Y. Chuang, C.L. Chen, C.Y. Chiu, Biotechnol. Technol. 8 (1994) 447.
[148] P.F. Ruhn, S. Garver, D.S. Hage, J. Chromatogr. A 669 (1994) 9.
[149] A.D. Denizli, E. Piskin, J. Chromatogr. B 666 (1995) 215.
[150] W.C. Lee, C.Y. Chuang, J. Chromatogr. A 721 (1996) 31.
[151] A. Serres, E. Legendre, J. Jozefonvicz, D. Muller, J. Chromatogr. B 681 (1996) 219.
[152] D. Müller-Schulte, S. Manjini, M.A. Vijayalakshmi, J. Chromatogr. 539 (1991) 307.
[153] E. Bisse, H. Wieland, J. Chromatogr. 575 (1992) 223.
[154] F.B. Anspach, J. Chromatogr. A 672 (1994) 35.
[155] A. Serres, D. Muller, J. Jozefonvicz, J. Chromatogr. A 711 (1995) 151.
[156] A. Denizli, B. Salhi, E. Piskin, J. Chromatogr. A 731 (1996) 57.
[157] T. Koyama, K. Terauchi, J. Chromatogr. B 679 (1996) 31.
[158] I.Y. Galaev, B. Mattiasson, Bio/Technology 12 (1994) 1086.
[159] I.Y. Galaev, N. Gard, B. Mattiasson, J. Chromatogr. A 684 (1994) 45.
[160] I.Y. Galaev, B. Mattiasson, J. Chromatogr. A 662 (1994) 27.
[161] I.Y. Galaev, B. Mattiasson, J. Chromatogr. A 684 (1996) 37.
[162] I.Y. Galaev, B. Mattiasson, Enzyme Microb. Technol. 15 (1993) 354.
[163] W. Jacques, M.A. Mateescu, Anal. Lett. 26 (1993) 875.

[164] C. Harayama, M. Sakata, S. Morimoto, H. Tachibe, H. Ihara, K. Ohkuma, Y. Miyachi, Chromatographia 40 (1995) 33.
[165] C. Harayama, M. Sakata, Y. Yugawa, H. Ihara, J. Chromatogr. A 676 (1994) 267.
[166] A.L. Dawidowicz, D. Wasilewska, S. Radkiewicz, Chromatographia 42 (1996) 49.
[167] B. Lorenz, S. Marme, W.E.G. Müller, K. Unger, H.C. Schröder, Anal. Biochem. 216 (1994) 118.
[168] K.A. Giuliano, Anal. Biochem. 200 (1992) 370.
[169] M.H. Glavanovich, P.W. Carr, Anal. Chem. 66 (1994) 2584.
[170] J.L. Liao, Y. Wang, S. Hjerten, Chromatographia 42 (1996) 259.
[171] N. Nimura, H. Itoh, T. Kinoshita, J. Chromatogr. A 689 (1995) 203.
[172] K. Hosoya, Y. Kishii, K. Kimata, T. Araki, N. Tanaka, Chromatographia 38 (1994) 177.
[173] V. Smigol, F. Svec, J.M.J. Fréchet, J. Liq. Chromatogr. 17 (1994) 891.
[174] D. Berek, I. Novak, M. Petro, J. Radioanal. Nuclear Chem. 183 (1994) 19.
[175] D.C. Sherrington, Macromol. Chem., Macromol. Symp. 70/71 (1993) 303.